PCT

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : **C12N 15/10, C12Q 1/68**	**A1**	(11) International Publication Number: **WO 95/05461** (43) International Publication Date: 23 February 1995 (23.02.95)

(21) International Application Number: PCT/US94/08498

(22) International Filing Date: 4 August 1994 (04.08.94)

(30) Priority Data:
08/107,440   13 August 1993 (13.08.93)   US

(71) Applicant: E.I. DU PONT DE NEMOURS AND COMPANY [US/US]; 1007 Market Street, Wilmington, DE 19898 (US).

(72) Inventor: RAKESTRAW, Scott, Lawrence; 8 Spring Hill Lane, Newark, DE 19711 (US).

(74) Agents: GEIGER, Kathleen, W. et al.; E.I. du Pont de Nemours and Company, Legal/Patent Records Center, 1007 Market Street, Wilmington, DE 19898 (US).

(81) Designated States: AM, AU, BB, BG, BR, BY, CA, CN, CZ, FI, GE, HU, JP, KG, KP, KR, KZ, LK, LT, LV, MD, MG, MN, NO, NZ, PL, RO, RU, SI, SK, TJ, TT, UA, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD).

Published
With international search report.

(54) Title: *IN SITU* EXTRACTION OF MICROBIAL DNA

(57) Abstract

A method for the extraction of microbial DNA which renders such DNA amenable for further enzymatic modification *in situ*, including digestion of the DNA with restriction endonuclease. The method comprises: a) heating the microbial cells in the presence of a hypotonic EDTA solution, b) lysing the cells by addition of achromopeptidase and c) heating said lysed cells to inactivate remaining protease and nuclease.

TITLE

IN SITU EXTRACTION OF MICROBIAL DNA

FIELD OF THE INVENTION

This invention relates to a method for *in situ*

5 lysis of bacterial cells and subsequent enzymatic
modification of the bacterial DNA. The method does not
require specific isolation, purification, or separation
of the DNA from the bacterial lysate prior to enzymatic
modification.

10   BACKGROUND OF THE INVENTION

Within the rapidly advancing field of microbial
diagnostics, research and analytical procedures now
require analysis at the nucleic acid level to provide
the ultimate identification code unique to each specific

15 microbial strain.

Numerous extraction methods have been developed in
attempts to efficiently access these nucleic acids. For
diagnostic systems entailing the analysis of a variety
of unknown microbes, the method of choice must be

20 capable of simultaneously extracting nucleic acids from
a range of bacterial types, including both Gram negative
and Gram positive bacteria. Further, a useful method
must render extracted nucleic acids which are
sufficiently intact and in suitable condition for

25 further biochemical analysis and enzymatic
characterization.

U.S. Patent No. 4,900,677 and European Patent
Application 87308431.3 (Hewitt) disclose a procedure for
isolating high molecular weight nucleic acids utilizing

30 a mixture of lytic enzymes in combination with a
chaotropic agent. Unlike the instant invention, the
method necessarily entails a dialysis and concentration
step to remove enzyme inhibitors before the extracted
nucleic acids may be analyzed enzymatically.

International Patent Application No. PCT/US89/0346
(WO90/102179, Lifecodes Corporation) discloses a broad
process for isolating DNA from a range of biological
samples, including blood cells, semen, urine, tissue,
5   Hela Cells, hair and *E. coli*. The method states that a
separate purification step to remove residual
proteolytic activity from lysed cells may be avoided by
heating the lysed samples to a temperature and for a
time sufficient to autodigest or inactivate residual
10   proteolytic activity. Unlike the instant method,
however, this disclosure does not enable a method which
has utility in extracting nucleic acids from both Gram
negative and Gram positive bacterial strains. Further,
in contrast to the instant invention, this diclosure
15   does not demonstrate efficacy in extracting nucleic
acids which are suitable for direct enzymatic
manipulation *in situ*.

                    SUMMARY OF THE INVENTION
This invention provides a process useful for the
20   extraction or purification of nucleic acids from
microbial cells. The invention is effective in
extracting intact nucleic acids from a broad range of
bacteria, including Gram negative and Gram positive
strains. The extraction method is performed *in situ*,
25   and yields nucleic acids which are able to be further
enzymatically manipulated *in situ*.

Specifically, the invention provides an *in situ*
method to extract nucleic acids from microbial cells
comprising the steps of (a) heating the microbial cells
30   in the presence of a hypotonic EDTA solution at a
temperature and time period sufficient to deactivate
endogenous cellular nuclease activity; (b) lysing the
microbial cells by addition of achromopeptidase in
hypotonic solution and incubating said lysed cells for a
35   time sufficient to inactivate internal cellular

degradative factors; and (c) heating said mixture of
lysed cells to a temperature sufficient to inactivate
remaining protease and nuclease activity; to yield
extracted microbial double-stranded nucleic acids which

5   are suitable for further *in situ* enzymatic manipulation.

In a preferred embodiment, microbial cells are
heated to 80°C for 10 minutes in hypotonic 20 mM EDTA;
and then lysed by incubation with added achromopeptidase
for 25 minutes at 37°C. The lysed cells are then

10 incubated for 25 minutes at 70°C to deactivate residual
proteolytic or nuclease activity.

In a most preferred embodiment, extracted microbial
DNA is then enzymatically treated with a restriction
endonuclease within the same cellular extraction vessel,

15 and the restriction digest is electrophoretically
chromatographed on a gel whereby the resulting
restriction pattern is visualized and compared against
known patterns as a means to identify an unknown strain
of bacteria.

20   BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows the electrophoretic pattern of an
agarose gel of Gram positive and Gram negative bacterial
DNA which was extracted using the disclosed method and
digested with the restriction endonuclease EcoRI,

25 labeled with a DNA probe; and visualized using
chemiluminescent labeling.

              DETAILED DESCRIPTION OF THE INVENTION

Described herein is a method for the *in situ*
isolation and optional further *in situ* enzymatic

30 manipulation of nucleic acids, preferably DNA, derived
from microorganisms, preferably bacteria. This method
comprises:

              (a)   thermally treating the microorganism
cells to deactivate the endogenous nuclease activity

35 associated with the cells;

(b)   lysing the microorganism cells to release
the component DNA and incubating the DNA to render it
amenable to subsequent enzymatic action; and

(c)   deactivation of residual nuclease and
5   protease activity by thermally treating to denature
remaining nucleases and proteases.

Optionally,

(d)   further enzymatic modification of the DNA
in the same reaction vessel may be performed.

10   In the present invention, steps (a)-(d) are made to
occur without intermediate isolation, separation, or
purification of the DNA and are effective on both Gram
positive and Gram negative bacteria. Furthermore, it is
an advantage of the present invention that the process
15   can be performed in a single vessel. It is yet another
advantage of the present invention that the process can
be performed entirely with reagents supplied in the
liquid phase. Thus, the method is easily automatable.

For purposes of the present disclosure, Applicant
20   intends that the following terms convey the meanings set
forth below.

In situ is used throughout to indicate that the
total method described (i.e., the extraction of
microbial DNA and optional further enzymatic digestion)
25   can be carried out within a single vessel.

The term "DNA" refers to native, double-stranded
deoxyribonucleic acids. For convenience, Applicant
throughout the text refers to DNA or nucleic acids
interchangeably, but intends to include all classes of
30   nucleic acids, such as RNA and DNA from any source
within a microbial cell.

Microbial cells includes all classes of Gram
positive and Gram negative bacteria.

Hypotonic refers to any solution possessing an
35   osmolality of dissolved solutes below that which is

5

commonly observed for the interior of intact microbial
cells.

By the term "extraction", as used in the phrase
"*in situ* extraction of nucleic acids from cells",
5   Applicant means that the microbial nucleic acids become
disassociated from their natural intracellular packaging
while remaining intact in a native, double-stranded
form. The extraction renders the nucleic acids
accessible in solution for further manipulation.
10   By the term "suitable for further *in situ* enzymatic
manipulation", Applicant refers to the fact that the
extracted DNA is now accessible for digestion with
enzymes which may be added to the same reaction vessel.
Applicant also specifically contemplates and includes
15   within this descriptive phase that such extracted
nucleic acids are also rendered accessible and suitable
for procedures other than direct enzymatic
manipulations, such as labeling or characterization by
other means known in this art.
20   The invention is particularly described and
discussed in the following set of steps which are set
forth, for purposes of illustration only, using
bacterial DNA which is extracted and then further
digested with the restriction endonuclease EcoRI.
25   (a)   <u>Deactivation of the Endogenous Nucleases</u>. The
first step in the method is heat treatment of the intact
bacterial cells, which in combination with the presence
of hypotonic EDTA (approximately 20 mM), causes
disruption of cell walls, and inactivation of the
30   endogenous nucleases. Failure to initially inactivate
the endogenous nucleases frequently results in
spontaneous degradation of the bacterial DNA during
further processing of the cellular material. Applicant
has found particularly that Gram negative cells (in
35   particular *Salmonella* spp.) were observed to display

extensive endogenous nuclease activity in the absence of
adequate heat treatment. The optimal time-temperature
treatment was found to be 80 ± 2°C for 10 minutes in
basal lysis buffer (1mM Tris, 20 mM EDTA, pH 8.0).

5   While heat treatments for less than 10 minutes at 80°C
did yield adequate results for some cell lines
(particularly the Gram positive cell lines), such
treatments were determined to fail for a significant
proportion of cell lines under study. In a similar

10   manner, treatment temperatures less than 80 ± 2°C for
10 minutes did not deactivate the endogenous nucleases
for all cell lines studied. Treatment temperatures in
excess of 80°C led to rapid denaturation of the double-
stranded DNA contained in the bacterial genome, and

15   inability to produce the subsequent restriction digest.
Failure to heat treat the cells at all led to severe DNA
degradation for most cell lines studied.

Integral to deactivation of endogenous nucleases is
the presence of EDTA. The EDTA chelates the divalent

20   cations which are known to stabilize the organization of
the bacterial cell wall. Furthermore, almost all of the
endogenous nucleases in the bacterial kingdom are known
to be metalloenzymes dependent on divalent cations for
their activity. Insufficient EDTA allows the cell wall

25   to remain stable to lytic enzymes, and preserves
endogenous nuclease activity. The optimal concentration
of EDTA for the range of reagents used generally was
found to be 20 mM in the presence of 1 mM Tris at
pH 8.0. In this system, below a concentration of 5 mM

30   EDTA, DNA degradation was observed for most cell lines.
Above a concentration of 30 mM EDTA, complications with
the activity of the enzyme ACP were noted.

(b)   Lysis of the Bacterial Cells. The second step
of the method is incubation of the cells with the

35   bacteriolytic peptidase achromopeptidase (ACP), which

causes rupture of peptide bonds in the cell wall leading
to a loss of integrity of the cellular structure.
Because this procedure is performed under hypotonic
conditions, disruption of the cell wall leads to cell

5   lysis as a result of the high internal osmotic pressure
gradients found in bacterial cells.

        The peptidolytic enzyme achromopeptidase (ACP) has
been known to possess bacteriolytic activity for some
time.   Previous reports have focused on the lytic

10   activity of the enzyme for Gram positive cells. In the
present work, suprisingly, Applicant has determined that
the enzyme functions equally well as a general
bacteriolytic enzyme under appropriate conditions. The
stock concentraction of ACP was varied in the range 250-

15   4000 Units/ml of crude preparation (Wako Chemical, Inc.,
Richmond, VA) in deionized water solution. Below a
stock concentration of 500 Units/ml the ACP
concentration was found to be insufficient to cause
general lysis of bacteria in less than 45 minutes

20   (37°C). At stock ACP concentration of ≥500 Units/ml,
bacterial lysis was determined to be complete in
25 minutes for all cell lines studied. However, above
stock ACP concentration of 1000 Units/ml the residual
proteolytic activity of the ACP required extensive heat

25   treatment in order to inactivate the enzyme prior to
introduction of the restriction endonuclease. Preferred
mode is to use a stock concentration 500 Units/ml
because such concentration provided rapid bacterial
lysis (t=25 minutes at 37°C) without undue difficulty in

30   the abolishment of residual proteolytic activity in the
next process step.

        A target time of 30 minutes or less was found to be
optimal for the lysis step at 37°C. In light of this
goal, an ACP concentration of 500 Units/ml was found to

35   be sufficient to cause complete bacterial lysis in

25 minutes at 37°C. Lytic incubation times less than
25 minutes must be avoided as they were found to yield
incomplete lysis for some bacterial cell lines studied.
Incubation for up to 60 minutes at an ACP concentration
5   of 500 Units/ml and 37°C was not noted to adversely
affect the process.

Product literature for the enzyme (Wako Chemical,
Inc., Richmond, VA) indicates that several cations are
known inhibitors of the enzymatic activity. These
10 inhibitors are summarized in the table below. In
addition, the present study found that total cation
concentrations exceeding 25 mM should be avoided for any
cation. For these reasons, crude ACP preparations must
always be prepared in deionized water.

15 Because ACP is a peptidase, it will display non-
specific proteolytic activity, including autodigestion.
ACP should thus be stored in the dry state at 4°C or
frozen (-20°C) in deionized water in order to avoid
autodigestion of the enzyme. Under either of these
20 conditions, no appreciable loss of enzyme activity for
periods up to one year have been observed.

Some proteins were found to interfere with the
action of ACP. Prior to the current embodiment, the
process used a cocktail of both ACP and lysozyme to
25 affect the lysis of bacterial cells. The cationic
nature of lysozyme at physiological pH was found to
inhibit the activity of ACP.

Known Inhibitors of Achromopeptidase Activity

Reagent	Percent Relative ACP Activity at 10^{-3} M	Percent Relative ACP Activity at 10^{-4} M
None	100	100
Ca^{++}	51	88
Mg^{++}	54	91
Ba^{++}	51	82

Mn^{++}	33	91
Zn^{++}	0	0
Cd^{++}	5	15
Hg^{++}	0	0
Fe^{++}	5	56
Co^{++}	16	41
Ni^{++}	21	56
Pb^{++}	16	62
Cu^{++}	0	10
Ag^{++}	34	48
Al^{+++}	0	56
EDTA	100	--
2-Mercaptoethanol	95	--
Sodium Azide	100	--
SDS	53	--

Data from Wako Chemical, Inc. (Richmond, VA)

   Treatment of the DNA to Render it Amenable to
Subsequent Manipulation. Once the DNA has been released
from the lysed cells, it must be treated to remove bound
5 proteins and other cellular debris before it can be
digested with a restriction endonuclease. Due to its
native peptidolytic activity, ACP is thus employed as a
double agent to both lyse the cells as well as degrade
proteinaceous substances that may interfere with
10 subsequent processing. No further reagent additions are
needed to allow ACP to perform its proteolytic task.
This process step occurs simultaneously with the
bacterial lysis. Incubation of the cell lysate at 37°C
promotes the action of ACP.
15   (c) Deactivation of Residual Nuclease and Protease
Activity. Before the bacterial DNA can be enzymatically
digested with for example, a restriction endonuclease,
residual endogenous nuclease and protease activity must
be abolished. Treatment of the cell lysate at high

temperature (70±1°C) is used to accomplish the goal.
Such treatment leads to thermal denaturation of the
remaining nucleases and proteases. Particularly, the
beneficial ACP proteolytic activity employed to strip

5   the DNA of bound proteins during lysis must be removed
before the restriction digest can proceed. Because
external digestive enzymes such as EcoRI will
subsequently be introduced in very small amounts, any
residual proteolytic activity would rapidly destroy

10 external enzymatic activity and result in undigested or
partially digested DNA. Remaining protease activity was
found to be abolished by the action of heat to thermally
denature the proteases. Temperature is of the utmost
importance in this operation. The optimal temperature

15 was determined to be 70 ± 1°C. Temperatures above 71°C
were observed to melt the double-stranded genomic DNA,
resulting in incomplete restriction digests and spurious
smeared DNA bands as observed by agarose gel
electrophoresis. Temperatures below 69°C resulted in

20 inefficient destruction of the protease activity. Below
65°C protease activity could not be destroyed in less
than 60 minutes of incubation time. Because a target of
≈30 minutes was set for the protease deactivation time,
an optimal condition of 70 ± 1°C for 30 minutes was

25 determined.

        (d)   Optionally, the extracted DNA may now be
further enzymatically digested *in situ*. One example is
the digestion of the lysate with the restriction
endonuclease EcoRI to generate a spectrum of DNA

30 fragments. In this embodiment, excess magnesium ions
must be added to overcome the chelation inhibition of
the EDTA used in step (a). In addition to the
requirement for appropriate buffer conditions, RNAse A
is added to the reaction mixture during this step in

35 order to degrade bacterial RNA. Failure to degrade the

bacterial RNA can cause spurious signal to be observed
after electrophoretic processing, and in extreme cases,
can cause inhibition of the activity of the restriction
endonuclease.

5   In one most preferred embodiment of the method, the
resulting restriction digest is further analyzed by gel
electrophoresis and DNA probe methods to generate a band
pattern which may be used to identify or characterize
the bacteria [US Patent No. 4,717,653 (Webster); US

10   Patent No. 5,087,558 (Webster); US Patent No. 4,885,697
(Hubner)]. The restriction digest is first terminated
by the addition of the loading buffer. The loading
buffer contains EDTA, which stops the digestion
reaction. Moreover, the loading buffer contains a

15   densifying agent (e.g., Ficoll, sucrose) in order to
prepare the digested DNA for loading onto an agarose
separation gel. The agarose gel electrophoresis may
then proceed by routine methods well known in the art,
to yield an electrophoretic pattern of bacterial DNA

20   fragments.

ALTERNATIVE UTILIZATION OF THE EXTRACTED DNA

        While the above-described process for Applicant's
method includes a restriction endonuclease digestion of
DNA as the last step, Applicant believes the disclosed

25   process is also suitable for performing a variety of
other manipulations of the harvested DNA. Each
modification (with the exception of PCR) described below
may be inserted in the claimed method after the high
temperature deactivation of step (c). Examples of such

30   enzymatic manipulations and procedures include:

        Performance of DNA amplification reactions. A
variety of nucleic acid amplification procedures could
be employed after step (c) in the above procedure
including, for example: polymerase chain reaction (PCR,

35   U.S. 4,683,195); ligase chain reaction (LCR, EPO

12

Publication No. 0 473 155 A2); or random amplified
polymorphic DNA analysis (RAPD, U.S. 5,126,239).
Addition of the desired polymerases (or ligases, etc.),
appropriate primers, deoxynucleotides, and a suitable
5   reaction buffer to the DNA generated will allow DNA
amplifications to proceed in the usual manner. Because
some amplification reactions require high temperatures
($\geq$ 75°C) as an integral part of the process, it may be
possible to obviate the need for a separate high
10   temperature step (c) in the basic method.

     Other enzymatic operations performed on the DNA. A
variety of other DNA manipulation enzymes are routinely
used in molecular biological work. For example, one
might employ ligases, DNA polymerases, phosphatases,
15   endo- or exo- nucleases, non-specific DNAses, or other
enzymes which can label or modify DNA. Furthermore,
transcription enzymes (e.g., T7 or SP6 RNA polymerase)
may be used to transcribe the DNA template into
corresponding RNA copies in the presence of the
20   appropriate nucleotides and buffers.

     Labeling of DNA substrates for the purpose of
generating DNA probes. A common objective of molecular
biological work is the production of DNA probes which
are labeled for use as a nucleic acid detection agent.
25   For example, nick translation protocols are routinely
used to label the DNA substrates via enzymatic action.
In addition, labels containing chemically reactive
linking groups (e.g., Photobiotin, fluorescein
derivatives) may be added in situ to the DNA for the
30   purpose of labeling or otherwise modifying the DNA.

     Cloning experiments. Some molecular biology
experiments or processes seek to produce clonal
libraries of recombinant DNA. These libraries are
usually generated by (1) fragmenting the substrate DNA
35   with restriction endonucleases or other nucleases; (2)

adding the desired exogenous DNA insert fragment to the
fragmented substrate DNA; (3) ligating the insert
fragment to the substrate fragments with ligases; and
(4) screening the recombinant DNA produced via probing
of the DNA extracted from clones carrying a particular
insert. Most of the steps involved in a cloning
experiment use enzymatic manipulations of DNA. Thus, a
clonal library may be constructed *in situ* using the
methodology described herein.

10   EXAMPLES

General Reagents

Basal Lysis Buffer   1 mM Tris, 20 mM Na$_2$EDTA, pH 8.0
Achromopeptidase   500 Units/ml (WAKO-crude fraction;
                            0.5 mg/ml) in deionized water
Digestion Buffer   160 Units/ml RNAse A (Sigma;
                            2 mg/ml), 280 mM Tris, 560 mM NaCl,
                            89 mM MgCl$_2$, 6 mM dithioerythritol,
                            pH 7.5
EcoRI Enzyme   50 Units/µl (Boehringer-Mannheim)
Loading Buffer   10% Ficoll (Pharmacia), 2 mM EDTA,
                            traces of bromophenol blue and
                            xylene cyanol added as tracking
                            dyes

EXAMPLE 1

This Example describes the lysis of a Gram negative
bacterial species (e.g., *E. coli*) and subsequent *in situ*
EcoRI digestion of DNA obtained from the cells (see
lanes 11, 12 and 13 of Figure 1). *E. coli* bacterial
colonies (5 x 10^7 to 5 x 10^8 cells) were first retrieved
from an overnight Petri plate and suspended in Basal
Lysis Buffer to an optical density of 0.40 (600 nm,
pathlength = 1.0 cm). An aliquot (30 µl) of the cell
suspension was added to a sealable reaction vessel, and
heated at 80 ± 1°C for 10 minutes in a waterbath. The
cell sample was allowed to air cool to 37°C.

14

Achromopeptidase (70 µl) was added with mixing to the
contents of the reaction vessel. The reaction vessel
was held for 25 minutes at 37 ± 2°C in order to permit
the lysis of the cells. The temperature of the reaction
5   vessel was then raised to 70 ± 1°C over a span of
7 minutes and then held at 70 ± 1°C for an additional
25 minutes. The purpose of this high temperature step
was to deactivate the residual proteolytic activity of
the achromopeptidase. Following the high temperature
10   step, the contents of the reaction vessel were allowed
to cool to 37 ± 2°C over a span of 6 minutes. At this
point, the reaction mixture is referred to as the
neutralized lysate.

In a separate vessel, 126 µl of EcoRI solution (in
15   the form supplied by the manufacturer, 50 Units/µl) was
thoroughly mixed with 576 µl of the Digestion Buffer.
An aliquot of this solution (23 µl) was then pipeted
into the neutralized lysate mixture from above with
thorough mixing. The restriction digestion of the
20   bacterial DNA contained in the neutralized lysate
proceeded for 20 minutes at 37°C. At this point, the
bacterial DNA had been digested into a spectrum of
characteristic fragments by the restriction endonuclease
EcoRI. The digestion reaction was terminated and the
25   DNA fragments were readied for electrophoretic analysis
by the addition of 10 µl of Loading Buffer. The samples
were then electrophoresed on a gel of 0.8% (w/w)
agarose, and electroblotted onto a nylon immobilization
membrane. The blotted DNA was then contacted with a
30   conserved sequence ribosomal DNA probe as described in
US patents 4,717,653 and 5,087,558 (Webster), which are
hereby incorporated by reference in their entirety. The
probed DNA bands were then visualized using a
chemiluminescent system as disclosed in allowed patent
35   application U.S. Serial No. 07/739,123, which is hereby

incorporated by reference. The gel image was then
captured (Figure 1) using a digital CCD camera by
techniques well known in the art.

      Alternatively, if the DNA fragments are to be used
5   for purposes other than electrophoretic analysis, the
digestion reaction may be terminated by the addition of
EDTA alone, or by rapid freezing with subsequent storage
at -20°C.

<u>EXAMPLE 2</u>

10   This Example describes the lysis of Gram positive
bacterial species (e.g., *Staphylococcus* spp., and
Listeria spp.) and subsequent *in situ* EcoRI digestion of
DNA obtained from the cells. The extraction,
electrophoresis, probe and visualization procedures were
15   conducted exactly as described in Example 1 except with
the incorporation of the desired Gram positive strain as
the bacterial sample (see lanes 1-10 of Figure 1).
Thus, the general utility of applicant's process in
extracting DNA from both Gram positive and negative
20   bacterial species was demonstrated.

<u>Legend of Figure 1, for Examples 1 and 2</u>

Lane #	Sample
1	Image Marker Standard
2	*Listeria ivanovii*
3	*Listeria ivanovii*
4	Image Marker Standard
5	*Listeria monocytogenes*
6	*Listeria monocytogenes*
7	Image Marker Standard
8	*Staphylococcus aureus*
9	*Staphylococcus aureus*
10	Image Marker Standard
11	*Escherichia coli*
12	*Escherichia coli*
13	Image Marker Standard

What is claimed is:

1.   An *in situ* method to extract nucleic acids
from microbial cells, comprising the steps of:

(a)   heating the microbial cells in the
presence of a hypotonic EDTA solution at a temperature
and time period sufficient to deactivate endogenous
cellular nuclease activity;

(b)   lysing the microbial cells by addition of
achromopeptidase in hypotonic solution and incubating
said lysed cells for a time sufficient to inactivate
internal cellular degradative factors; and

(c)   heating said lysed cells to a temperature
sufficient to inactivate remaining protease and nuclease
activity;
to yield extracted microbial double-stranded nucleic
acids which are suitable for further *in situ* enzymatic
manipulation.

2.   The method of Claim 1 wherein said microbial
cells are comprised of Gram negative or Gram positive
bacterial cells.

3.   The method of Claim 1 wherein at step (a) said
heating is carried out at a temperature of 80 ± 2°C for
a period of about 10 minutes.

4.   The method of Claim 3 wherein at step (a) EDTA
is present at a concentration of about 20 mM in a
hypotonic solution of about 1 mM Tris at about pH 8.0.

5.   The method of Claim 1 wherein at step (b) said
lysis is carried out by addition of about 35 Units of
achromopeptidase (from a stock soultion of 500 Units/ml)
in a hypotonic solution, for a period of about
25 minutes at a temperature of about 37°C.

6.   The method of Claim 1 wherein at step (c) said
heating is carried out at a temperature of 70 ± 1°C for
a period of about 30 minutes.

7. The method of Claim 2, further comprising after step (c):

(d) performing *in situ* enzymatic modification of said extracted nucleic acids.

8. The method of Claim 7 wherein said *in situ* enzymatic modification comprises performing restriction digestion with a restriction endonuclease.

9. The method of Claim 8 wherein said restriction digestion is performed using the restriction endonuclease EcoRI.

10. The method of Claim 7 wherein said *in situ* enzymatic modification comprises replication of one or more sequence segments of said bacterial DNA.

11. The method of Claim 10 wherein said replication is performed using the polymerase chain reaction or random polymorphic DNA analysis.

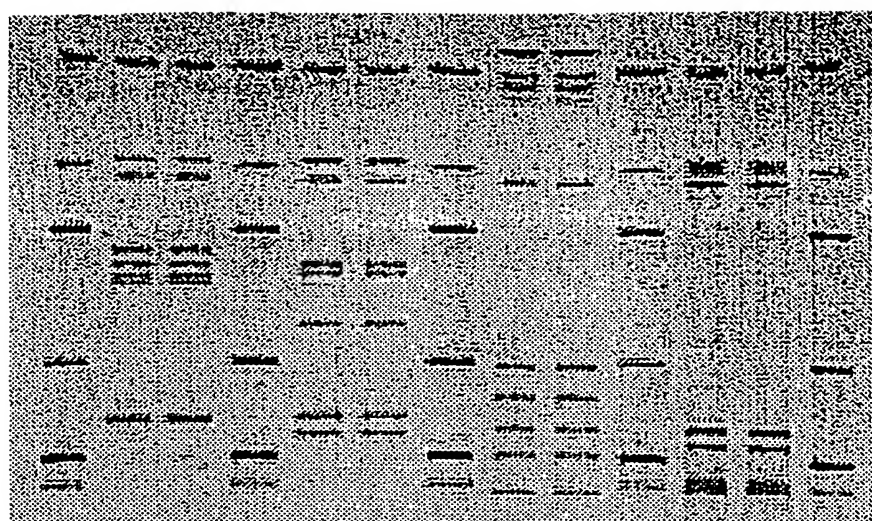12. The method of Claim 8 or 10 further comprising after step (d):

(e) analyzing the product of said enzymatic modifications.

13. The method of Claim 12 wherein said analysis is performed on the basis of size separation.

14. The method of Claim 13 wherein said separation is achieved through gel electrophoresis.

15. The method of Claim 12 wherein said analysis is performed by visualizing or detecting said enzymatically modified DNA using labels or dyes.

LANE #   1 2 3 4 5 6 7 8 9 10 11 12 13

FIGURE

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6   C12N15/10   C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6   C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US,A,4 830 969 (HOLMES D.) 16 May 1998 see column 2, line 54 - column 3, line 33 ---	1-5
A	EP,A,0 552 571 (BECTON DICKINSON AND COMPANY) 28 July 1993 see claims; tables I,II ---	1-5,10,11
A	EP,A,0 547 789 (BECTON DICKINSON AND COMPANY) 23 June 1993 see page 11, line 39 -----	6

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
8 November 1994	0 2 -12- 1994

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Van der Schaal, C

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
US-A-4830969	16-05-89	NONE		
EP-A-0552571	28-07-93	AU-A- CA-A-	3111393 2086729	15-07-93 10-07-93
EP-A-0547789	23-06-93	AU-A- CA-A-	2844892 2084533	24-06-93 19-06-93